JP97/040376 HAMA et al

FULL CONTENTS

[Claim(s)]

1. Biological Sample, Cholesterol Esterase of Pancreas Origin, and Cholesterol Oxidase, A bile acid or its salt is contacted under the conditions in which albumin exists, and it is characterized by measuring the compound consumed by the enzyme reaction of high-density-lipoprotein cholesterol in said biological sample, and each of said enzyme, or the compound generated. The measuring method of high-density-lipoprotein cholesterol.
2. Procedure according to claim 1 which albumin enforces under conditions in which 0.01 weight % or more of whole system of measurement exists.
3. Procedure according to claim 2 of adding albumin as reagent.
4. Furthermore, it is General Formula (I). : A-(CH2) N-CH3 (I)
They are the compound expressed with (A is a glucoside machine, a thio glucoside machine, a shook sirloin oxycarbonyl machine, or N-methyl glucamide carbonyl group among a formula, and n is the integer of 4-10), or a general formula (II). : B-CH2-CH(R1)-CH2-SO3- (II)
It is a procedure given in Claim 1 which makes at least one sort of a compound expressed with [B is 3-(3-cholamidopropyl) dimethylammonio machine among a formula, and R1 is a hydrogen atom or a hydroxy group] live together - any 1 clause of three.
5. Claim 1 whose biological sample is serum or plasma - procedure given in any 1 clause of four.
6. High-Density-Lipoprotein Cholesterol in Biological Sample, and Cholesterol Esterase, Contact cholesterol oxidase and the compound consumed by the enzyme reaction of said cholesterol and each of said enzyme or the compound generated is measured. The reagent constituent which is a reagent constituent for making high-density-lipoprotein cholesterol a fixed quantity specifically, and is characterized by containing cholesterol esterase of pancreas origin as albumin, a bile acid or its salt, and said cholesterol esterase.
7. Liquefied constituent containing pancreas origin cholesterol esterase, cholesterol oxidase, albumin, bile acid or its salt, and buffer solution according to claim 6.
8. Constituent according to claim 6 which consists of the liquefied first reagent containing albumin and buffer solution, and the liquefied second reagent containing pancreas origin cholesterol esterase, cholesterol oxidase, and buffer solution.
9. Furthermore, it is General Formula (I). : A-(CH2) N-CH3 (I)
They are the compound expressed with (A is a glucoside machine, a thio glucoside machine, a shook sirloin oxycarbonyl machine, or N-methyl glucamide carbonyl group among a formula, and n is the

integer of 4-10), or a general formula (II). : B-CH2-CH(R1)-CH2-SO3- (II)
B is 3-(3-cholamidopropyl) dimethylammonio machine among [type. The compound by which R1 is expressed with the constituent 10. aforementioned general formula (I) containing at least one sort of a compound expressed with] which is a hydrogen atom or a hydroxy group according to claim 6 or 7, Or the constituent according to claim 8 which contains at least one sort of a compound expressed with a general formula (II) into the first reagent and/or the second reagent.
11. The dry analysis element containing the reagent field which sank in and dried the constituent containing pancreas origin cholesterol esterase, cholesterol oxidase, albumin, a bile acid or its salt, and buffer solution.

[Detailed Description of the Invention]
The specific measuring method and the constituent technical field for measurement of HDL cholesterol
This invention relates to the specific measuring method and the constituent for measurement of high-density-lipoprotein (HDL) cholesterol.
Background art Importance is attached to the cholesterol contained in each lipid fraction in plasma or serum as a diagnostic material in which the danger of atherosclerosis or myocardial infarction is shown in recent years. The lipid fraction of serum differs in the size as lipid complex particles, respectively. According to the ultracentrifugal method which is a used separation method, the difference of specific gravity Chylomicrons, Very low density lipoprotein (Very low density lipoprotein; it is also called Following VLDL), It is classified by four kinds of low density lipoprotein (Low density lipoprotein; it is also called Following LDL) and high density lipoprotein (High density lipoprotein; it is also called Following HDL). Each lipid fraction is divided roughly into apolipoprotein and a lipid, and the lipid consists of separated type cholesterol, cholesterolester, a triglyceride, and phospholipid further. For this reason, measurement of cholesterol is performed about both separated type and ester type.
In the everyday clinical test, although measurement of the total cholesterol by enzymatic process was widely performed using the autoanalyzer, since it was required to pretreat a sample (fractionation and separation operation), about the lipid fraction, the spread of the automatic analysis measurement (automation) by enzymatic process was behind. Various sedimentation methods are performed as a pretreatment of this sample. For example, the phosphotungstic acid, ionized magnesium, dextran sulfate and ionized magnesium, A heparin, calcium ion, or manganese ion (-- M. -- Burstein and H.R.Scholnick, Adv.Lipid Res., 11 and 67, 1973; and G.R.Warnick etal., Clin.Chem., and 25, 596 and 1979 --) -- Or pressure of business of the procedure of adding a polyethylene glycol, settling LDL etc. and using a supernatant as a specimen by centrifugal operation is carried out. In detail, when the phosphotungstic acid and ionized magnesium are used as a precipitant, a sample (serum and plasma) is added to the solution containing these, and let lipid fractions other than HDL be insoluble complexes. Except for precipitation, the supernatants containing HDL are collected by carrying out centrifugal separation of this. Measurement of HDL by which fractionation was carried out by an automatic analysis system is attained with the enzyme reagent for total measurement cholesterol.
Moreover, also in the immunization (C-C.Heuck, Clin.Chem., 31, 252, 1985), the immune body to apolipoprotein B (not contained in HDL) is added to a sample (serum and plasma) as a precipitant, and lipid fractions other than HDL are settled. After carrying out fractionation like the following, HDL content cholesterol in a supernatant can be measured for the first time. Thus, there was a fault that the

conventional procedure took each much processes and time.
The report is issued these days about the measuring method which does not need these fractionation operations (for example, a JP,6-16720,B number, a JP,7-34760,B number, or JP,58-165800,A No. each gazette). namely, as enzymatic process for the total measurement cholesterol used conventionally Cholesterol esterase hydrolyzes a cholesterol ester. Cholesterol oxidase is made to act on cholesterol which is this enzyme reaction product. Under existence of a suitable oxidizability color fixative, make the hydrogen peroxide which is made oxidized using a dissolved oxygen and is generated color it by a peroxidase reaction, and [carry out colorimetric measurement or] Or the procedure of measuring the dissolved oxygen amount consumed in the case of the oxidation reaction by the aforementioned cholesterol oxidase with an oxygen electrode was known.
For example, according to the description of each aforementioned patent journal, it is supposed that existence of the polyethylene oxide machine content surface-active agent of a non-ion system is important for the activity manifestation of cholesterol esterase with bile salt in the aforementioned system of reaction, and activity will not be discovered unless this surface-active agent exists. Moreover, about bile salt, the intervention to an activity manifestation is already clear in refining of the enzyme concerned (J. Hynn et al., JBC, 244 and 1937, 1969;, and KB.Calame et al.).
Arch.Biochem.Biophys., 168, 57, 1975.
And to JP,H6-16720,B [this bile salt] Since it is effective in making the cholesterol which a lipid melts only the chylomicron which is the lipoprotein which has comparatively slight protein in Toyotomi, and VLDL and LDL, and contains in it participate in an enzyme reaction, The procedure of carrying out fractionation measurement of the HDL cholesterol specifically is indicated by by taking the initiative in measurement of HDL cholesterol, making this react, adding the aforementioned surface-active agent subsequently and making cholesterol and enzyme which are contained in an HDL fraction react. That is, lipid fractions other than HDL were made to react first, subsequently, it was made to react with an HDL fraction and both difference is read.
Moreover, to JP,H7-34760,B, the procedure of using cholesterol esterase of pancreas origin as cholesterol esterase is further indicated in the same system as the above. [a signal] although a measurement signal is proportional to the cholesterol content of an LDL fraction in the beginning since isolation of the cholesterol contained in an LDL fraction is first performed by this procedure It restricts to the absorbance variation within a time [a certain] (for example, within a time [from a reaction start to after dichotomy progress - 15 minute progress]) after going through fixed time from a reaction start, and the characteristic reactivity proportional to HDL cholesterol concentration is shown. Furthermore, the complex by an antigen-antibody reaction is made to form between apolipoprotein B which is the main composition protein of LDL or VLDL by adding the anti-LDL immune body to the system of reaction, and said immune body. The device which raises the singularity over an HDL fraction synthetically by checking a reaction with the enzyme concerned is performed.
However, these procedures had the inadequate action to the automatic analysis machine currently used every day on manufacture cost -- to a reagent, an immune body is newly added, or reaction time is required 20 minutes or more, and there is -- or measurement operation.
On the other hand, H.Sugiuchi and others (Clin.Chem., 41, 717, 1995) has reported the new procedure which was adapted for the automatic analysis machine. That is, the enzyme which made combine it and carried out chemical modification of the polyethylene glycol to the enzyme (cholesterol esterase and cholesterol oxidase) concerned to be used, and polymer-ized it to it is used. Furthermore, it is making

the cyclodextrin inductor (specifically sulfation alpha-cyclodextrin) it is supposed that there are various lipid fractions and compatibility live together in addition to the aforementioned polymer-ized enzyme. It is indicated that a complex can be made to form to lipid fractions other than HDL. Since this complex cannot undergo the reaction by said polymer-ized enzyme easily, it can measure an HDL fraction specifically. However, when such a means is adopted, a modification of enzyme will accompany new problems, such as an increase in a new process, management of the degree of refining of an enzyme preparation and control of the enzyme activity change by the grade difference of chemical modification, management, and also maintenance of the stability of a modification enzyme.

this invention person etc. sets for the measuring method of HDL cholesterol in the present clinical test examination in view of the point that measurement by the autoanalyzer which is a quick and simple means is mainstream. It aims at development of the procedure by which it is simple operation and a highly precise measurement result is obtained, without performing centrifugal operation of a sample (serum or plasma). As a result of repeating research wholeheartedly, it is related with the reaction of the enzyme (cholesterol esterase and cholesterol oxidase) and lipid fraction content cholesterol which are used in the measuring method of HDL cholesterol. By finding out that the compound which checks a reaction with cholesterol of LDL and a VLDL fraction exists although a reaction with cholesterol of an HDL fraction is not influenced, and using these compounds By simple operation, it found out that HDL cholesterol could be measured with high precision, without performing centrifugal operation of a sample (serum or plasma). This invention is based on such knowledge.

Indication of invention This invention Therefore, a biological sample and cholesterol esterase of pancreas origin, Cholesterol oxidase, and a bile acid or its salt is contacted under the conditions in which albumin exists. It is related with the measuring method of the high-density-lipoprotein cholesterol characterized by measuring the compound consumed by the enzyme reaction of high-density-lipoprotein cholesterol in said biological sample, and each of said enzyme, or the compound generated.

This invention Moreover, high-density-lipoprotein cholesterol in a biological sample, Contact cholesterol esterase and cholesterol oxidase and the compound consumed by the enzyme reaction of said cholesterol and each of said enzyme or the compound generated is measured. It is a reagent constituent for making high-density-lipoprotein cholesterol a fixed quantity specifically, and is related also with the reagent constituent characterized by containing cholesterol esterase of pancreas origin as albumin, a bile acid or its salt, and said cholesterol esterase.

Brief explanation of the drawings Drawing 1 is graph which shows cholic acid sodium or the albumin addition effect at the time of using an LDL fraction as a specimen by the reaction variation per hour of enzyme and LDL.

Drawing 2 is HDL, LDL, VLDL, and the graph that shows the variation per hour of the absorbance of the normal pooled serum by this invention.

Drawing 3 is the chromatogram of the reaction mixture after making the reagent constituent and pooled serum of this invention react for 5 minutes.

Drawing 4 is the chromatogram of the reaction mixture after making the reagent constituent and pooled. serum of this invention react for 10 minutes.

Drawing 5 is graph which shows correlation of the HDL cholesterol measured value of this invention and a conventional method (sedimentation method).

Drawing 6 is graph which shows the albumin addition effect at the time of using an LDL fraction as a

specimen by the reaction variation per hour of enzyme and LDL.
Drawing 7 is graph which shows the albumin addition effect at the time of using serum as a specimen by the variation per hour of absorbance.
Drawing 8 is the chromatogram of the reaction mixture after making the auxiliary blocker content reagent constituent and pooled serum by this invention react for 5 minutes.
Drawing 9 is the chromatogram of the reaction mixture after making the auxiliary blocker content reagent constituent and pooled serum which do not contain albumin react for 5 minutes.
Drawing 10 is graph which shows correlation of the HDL cholesterol measured value of this invention (under auxiliary blocker existence), and a conventional method (sedimentation method).
Drawing 11 is graph which shows correlation of the HDL cholesterol measured value of this invention (dry type system of measurement) and a conventional method (sedimentation method).
The best form for inventing Especially in this invention, the serum or plasma of mammalian (especially Homo sapiens) can be used as it is as a biological sample. That is, it is not necessary to carry out fractionation processing by centrifugal operation etc., or to process serum or plasma by an immune body.
In this invention when contacting the aforementioned biological sample to cholesterol esterase and cholesterol oxidase As the compound which checks a reaction with LDL cholesterol and VLDL cholesterol, i.e., blocker, although the reaction of those enzymes and HDL cholesterol is not influenced, albumin is used.
In this invention, when a specimen is of blood (serum or plasma) origin, albumin is already contained in the specimen. However, albumin concentration of sample origin cannot fully acquire the effect by this invention to 0.01 or less weight % of a case in a system of measurement, i.e., the system which measures by mixing a reagent and a sample. When the albumin concentration of sample origin is high enough, it is not necessary to add artificially in particular but, and on the other hand, even if it adds albumin further, it is satisfactory in any way.
Moreover, to use as a specimen the refining lipid fraction and other living body liquid samples (for example, ****** or organization extract) which do not contain albumin, it is required to add and to make albumin live together. Thus, the albumin concentration in the case of adding albumin is 0.03 to 10 weight % more preferably 0.01 to 20weight %. The origin in particular of the albumin to add is not limited, for example, can also use the thing produced in genetic engineering besides the mammalian origins, such as a cow, Homo sapiens, a sheep, and a horse.
[cholesterol esterase and cholesterol oxidase which can be used in this invention] Especially, it cannot be limited, but the enzyme of the microbe origin or mammalian origin etc. can be used, and either the enzyme which was made to combine a polyethylene glycol (PEG) etc. and carried out chemical modification, or the enzyme which has not carried out chemical modification can be used.
In addition, when this invention person found out, in using the cholesterol esterase which has not carried out chemical modification in particular when using the enzyme which has not carried out chemical modification in this invention, reactivity changes with origins of the enzyme. It is more effective to choose the thing of the pancreas origin of mammalians, such as a cow or Buta, as cholesterol esterase preferably. Moreover, as cholesterol oxidase, the cholesterol oxidase of the microbe origin of Streptomyces or a NOKARUDEIYA group can be used, for example. setting to a system of measurement, although the amount of addition in particular of those enzyme is not limited, either -- for example, -- desirable -- 0.05u/ml-90u/ml -- more -- desirable -- 0.1u/ml-20u/ml -- it is.

When using cholesterol esterase of pancreas origin, it is desirable to use a bile acid or its salt simultaneously. The concentration is 0.15 - 1.5mM more preferably 0.05 to 4 mM in a system of measurement. Although the kind in particular of said bile acid or its salt is not limited, as a bile acid For example, a cholic acid, a taurocholic acid, a glycocholic acid, a chenodeoxycholic acid, a deoxycholic acid, or a lithocholic acid can be mentioned, and sodium salt etc. can be mentioned as the salt, for example. It is desirable that water solubility uses bile salt, for example, cholic acid sodium, the glycocholate sodium, or a sodium deoxycholate at a high point.
Moreover, it is a general formula (I) preferably [as auxiliary blocker] in this invention.
: A-(CH2)n-CH3 (I
They are the compound expressed with (A is a glucoside machine, a thio glucoside machine, a shook sirloin oxycarbonyl machine, or N-methyl glucamide carbonyl group among a formula, and n is the integer of 4-10), or a general formula (II). : B-CH2-CH(R1)-CH2-SO3- (II)
At least one sort of a compound expressed with [B is 3-(3-cholamidopropyl) dimethylammonio machine among a formula, and R1 is a hydrogen atom or a hydroxy group] can be made to live together.
the case where A is a glucoside machine or a thio glucoside machine in the compound expressed with said general formula (I) -- n -- desirable -- 4-9 -- it is 5-8 more preferably. a glucoside machine -- desirable -- a glucopyranoside machine -- it is a beta-D-glucopyranoside machine more preferably. a thio glucoside machine -- desirable -- a thio glucopyranoside machine -- it is a beta-D-thio glucopyranoside machine more preferably. Specifically as a compound in case A is a glucoside machine, an n-octyl beta-D-glucoside (it is also hereafter called n-ODG) and an n-heptyl beta-D-glucoside (it is also hereafter called n-HDG) can be mentioned. Moreover, specifically as a compound in case A is a thio glucoside machine, an n-octyl beta-D-thio glucoside (it is also hereafter called n-OTG) and an n-heptyl beta-D-thio glucoside (it is also hereafter called n-HTG) can be mentioned.
In the compound expressed with said general formula (I) when A is a shook sirloin oxycarbonyl machine n -- desirable -- 6-10 -- it is 7-9 more preferably and, specifically, a shook sirloin MONOKA plate (SM-1000 are called hereafter) can be mentioned as a compound in this case.
In the compound expressed with said general formula (I), when A is N-methyl glucamide carbonyl group, n is 5-9 preferably. As a compound in case A is N-methyl glucamide carbonyl group Specifically, octanoyl N-methyl glucamide (MEGA-8 are called hereafter), nonanoyl N-methyl glucamide (MEGA-9 are called hereafter), and decanoyl N-methyl glucamide (MEGA-10 are called hereafter) can be mentioned.
Specifically as a compound expressed with said general formula (II), it is 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (it is also hereafter called CHAPS) and 3-[(3-cholamidopropyl) dimethylammonio].
- 2-hydroxy 1-propanesulfonate (it is also hereafter called CHAPSO) can be mentioned.
In this invention, it is independent, or two or more sorts can be combined arbitrarily and the compound expressed with auxiliary blocker, i.e., the compound expressed with said general formula (I), or said general formula (II) can be used.
As for auxiliary blocker, in this invention, using as solution is desirable. The concentration of the auxiliary blocker in solution is 0.03 to 0.5 weight % 0.02 to 1.0weight % 0.01 to 2.0weight % preferably in a system of measurement. If there is less concentration of auxiliary blocker than 0.01 weight %, the prevention effect over an enzyme reaction with cholesterol of LDL and a VLDL fraction is not seen, and exact measurement cannot be performed. On the contrary, by the concentration exceeding 2.0 weight %,

the singularity over an enzyme reaction with cholesterol of a fraction is no longer seen at all, and it is inconvenient also in the soluble point of auxiliary blocker.
Even if it does not perform fractionation operation of centrifugal separation etc. beforehand before measurement about a specimen (for example, serum or plasma), according to this invention, under existence of albumin (blocker) While checking the reaction of the LDL cholesterol in a specimen and VLDL cholesterol, and enzyme by contacting a specimen (for example, serum or plasma) and enzyme (coexistence) It can be made to be able to go on without checking the reaction of HDL cholesterol and enzyme, a well-known means can detect the compound (for example, oxygen) consumed by the enzyme reaction of cholesterol and each of said enzyme, or the compound (for example, hydrogen peroxide) generated, and a fixed quantity of HDL cholesterol can be carried out. For example, when detecting hydrogen peroxide, H2O2 generated under existence of a suitable oxidizability color fixative and a peroxidase can be made to be able to color, and colorimetric measurement can be carried out in spectroscopy.
H2O2 are a well-known procedure and, for example, they can be made to color by the reaction of a peroxidase under existence of a suitable oxidizability color fixative. As an oxidizability color fixative, 3-high draw ****- 2 and 4, 6-triiodobenzoic acid (HTIBA), and N-ethyl N-sulfopropyl meta toluidine (ESPT) and 4-aminoantipyrine (4-AP) are suitable. For example, HTIBA and ESPT are the concentration ranges of 0.1mM - 5mM, and 4-AP can be made to contain suitably in the concentration range of 0.05mM - 2mM. What is necessary is just to measure the wavelength of 510nm (when using HTIBA), or the absorbance at 546nm (when using ESPT) in measurement by an autoanalyzer.
The reagent constituent for HDL cholesterol measurement by this invention contains cholesterol esterase of pancreas origin, cholesterol oxidase, albumin, and a bile acid or its salt. The reagent constituent for measurement by this invention can also contain further more than one sort or it of the compound expressed with said general formula (I) or said general formula (II) as auxiliary blocker by a case.
The reagent constituent for measurement by this invention preferably cholesterol esterase 0.05-90u/ml of pancreas origin -- [ml / 0.05 - 10u/] more preferably cholesterol oxidase 0.05-90u/ml -- [ml / 0.1 - 20u/] more preferably 0.01 to 20 weight % of albumin more preferably 0.02 to 10 weight %, a bile acid or its salt -- said 0.01 to 2.0 weight % of auxiliary blocker can contain 0.03 to 0.5 weight % more preferably by a case with 0.15 - 1.5mM 0.05 to 4 mM.
also being able to constitute and use the reagent constituent for measurement of this invention as a single reagent, and setting it by the autoanalyzer used widely now -- two reagents -- a system . [the case of a 2 reagent system, for example, albumin, albumin and one sort of auxiliary blocker, or the compound beyond it] respectively independent -- or it can live together, and can be made to be able to contain into either the first reagent or the second reagent, and the second reagent can be made to contain cholesterol esterase and cholesterol oxidase (in and the case a bile acid or its salt) As a buffer of a single reagent, the first reagent, and the second reagent, good buffer solution (for example, BES, HEPES, PIPES, or Bis-Tris etc.), a phosphate buffer solution, acetic acid buffer solution, Tris buffer, or an imidazole buffer can be used, for example. The concentration of buffer solution is 10 - 200mM five to 500 mM five to 1000 mM preferably. Moreover, pH of those buffer solution can be preferably chosen suitably within 4.5-8.0, and pH 5.5 to 7.5 more desirable limits with good prevention with cholesterol of LDL and cholesterol of VLDL, and enzyme.
It will be as follows if the system of reaction in the case of measuring HDL cholesterol is typically shown using the reagent constituent by this invention.

[Regulation of singularity]
Albumin and a bile acid, or its salt (it is auxiliary blocker by a case further)
+ Biological sample Substrate[non-]-izing of **LDL and VLDL [enzyme reaction and color reaction
(Cholesterol esterase reaction)
Cholesterol ester +H2O-> cholesterol + fatty acid (1)
(Cholesterol oxidase reaction)
Cholesterol +O2 ->delta 4-cholesten-3-one +H2O2 (2)
(Peroxidase reaction)
H2O2+ oxidizability color fixative -> oxidization condensation product (3)
** spectrometry In measurement by an autoanalyzer, H2O2 mainly generated by said reaction formula (2) can be measured with colorimetric method using the liquefied reagent constituent by this invention, and multi-specimen processing can be performed. This invention can be similarly used not only by a reaction in these solution but by the dry type system of measurement (dry chemistry) according to the piece of a filter paper test etc. for example. That is, apart from the multi-specimen processing by an automatic analysis machine, this invention can be used to an inspection demand individual as a simple measuring method. When applying this invention to the dry type system of measurement by transparent specimens, such as polystyrene, and performing measurement by the reflective type densitometry method, the usual transparent specimen can be infiltrated and the liquefied single reagent constituent by this invention prepared in suitable buffer solution can be manufactured with the conventional method to dry. Moreover, the element for dry analysis containing a development layer, a reagent layer, and a reflecting layer can be manufactured with the conventional method which makes a reagent layer contain the single reagent constituent by this invention. Furthermore, if the element for dry analysis which prepared independently the demarcation membrane or detached core of the corpuscle component in blood is used, blood can be directly measured as a sample as it is also in the institution which is not equipped with the separator machine for serum, or the separator machine for plasma.
[H2O2 to generate] in carrying out this invention By making it react under existence of suitable mediators, such as ferrocene, and a peroxidase directly, using a platinum electrode Or the variation of the oxidation current generated through suitable mediators, such as a potassium ferrocyanide, to direct cholesterol oxidase reaction time can also be measured electrochemically. On the other hand, the compound consumed by an enzyme reaction, for example, the oxygen consumed by said reaction formula (2), (dissolved oxygen) can also be conventionally measured by a well-known procedure, for example, an oxygen electrode. Moreover, as a compound generated by the enzyme reaction, you may measure delta 4-cholesten-3-one which is the product of said reaction formula (2) by a suitable procedure, for example besides the aforementioned hydrogen peroxide.
Although not limited to the following explanation, it sets to this invention. The reaction of the enzyme (cholesterol esterase and cholesterol oxidase) and lipid fraction content cholesterol which are used in the measuring method of HDL cholesterol is faced. [said auxiliary blocker which can live together by one or more sorts and case of albumin and a bile acid, or its salt] Compatibility is directly shown in the apolipoprotein of a lipid fraction, or to carry out an interaction to enzyme is indirectly considered by the reaction time of cholesterol of a lipid fraction, and enzyme.
Namely, although each lipid fraction serves as a lipid complex which consists of a lipid and apolipoprotein It is identified by the physicochemical quality and the quantitative (quantity contained in each fraction) difference arising from the difference of the difference in the lipid percentage, and the

types (A-1, A-1, B-100, B-48, C, or E) of apolipoprotein. Since the difference in the type (the former is A-1 or A-2, and the latter is B-100, C, or E) of apolipoprotein different most greatly between an HDL fraction, LDL, and a VLDL fraction is clear, The method of using the immune body to apolipoprotein has also been developed conventionally. It is the feature for this conventional method to mix with the immune body to the apolipoprotein B and C at a sample, and to make an immune complex form. Since only HDL cholesterol will react with enzyme if enzyme is added next in order to cause enzyme reaction prevention, this immune complex can measure only HDL cholesterol. However, difficultly, since muddiness of the immune complex itself was remarkable, maintaining uniformly the reactivity of the immune body which can form an immune complex had the fault that an error became large on the occasion of the colorimetric measurement for cholesterol measurement.

In aforementioned JP,H7-34760,B, an anti-LDL immune body and an anti-apolipoprotein B immune body are used. The method of raising the singularity of the reaction by cholesterol esterase of pancreas origin under existence of the surface-active agent of a bile acid group, a non-ion system surface-active agent, and cholesterol oxidase is indicated. Although use of these immune bodies is additional, as long as an immune complex is formed, same un-arranging arises. [the content] although the content of a setting of the reaction condition when not using an immune body is similar with a part of this invention The difference in the enzyme reaction characteristic by a bile acid or its salt living together by albumin and a case is essential, and it is clear by the following explanation that it is what is depended on the reaction mechanism from which both invention differs.

According to JP,H6-16720,B, the reaction of LDL cholesterol and VLDL cholesterol, and cholesterol esterase and cholesterol oxidase progresses under existence of bile salt first. Subsequently, since a reaction with HDL cholesterol starts by addition of the polyethylene oxide machine content surface-active agent of a non-ion system, the amount of HDL cholesterol shall be measured by searching for the difference of absorbance change obtained at the reaction of these 2 phase.

Moreover, according to the content of an indication of JP,H7-34760,B, it is shown like said JP,H6-16720,B under coexistence of the surface-active agent of a bile acid group, and a non-ion system surface-active agent that an enzyme reaction passes in two phases. A reaction with LDL cholesterol progresses first and the reaction with HDL cholesterol is started behind time in time. For this reason, it should choose suitably, for example, absorbance change in a predetermined time region shall be proportional to HDL cholesterol concentration within the limits of 2 to 15 minutes after a reaction start.

On the other hand, in this invention albumin and a bile acid or its salt, and by making one or more sorts of auxiliary blocker live together by a case further The conditions to which LDL cholesterol and VLDL cholesterol, and cholesterol esterase and cholesterol oxidase do not react existed, therefore it became clear that all the variation in arbitrary time is dependent on HDL cholesterol concentration from a reaction start. Especially in this invention, coexistence with albumin, a bile acid or its salt, and cholesterol esterase of pancreas origin can attain the purpose. It is as having already stated that cholesterol esterase of pancreas origin has indispensable bile acid or its salt to the activity manifestation. This reaction singularity can be easily investigated by, for example, comparing reactivity with each lipid fraction by which separation refinement was carried out from serum. Moreover, in order to investigate a reaction with serum more correctly, without separating each lipid fraction beforehand, it is directly verifiable by using a reaction mixture object chromatography method. The reaction with enzyme, LDL cholesterol, and VLDL cholesterol changes depending on the concentration of a bile acid or its salt, and its reaction is so early that concentration is high.

On the other hand, when albumin was added under existence of the bile acid of fixed concentration or its salt, the enzyme reaction received control in the albumin concentration dependence target, but at the reaction of enzyme and HDL cholesterol, it turned out that it is not controlled. Thus, the effect of the albumin which can give singularity to an enzyme reaction is the very specific character in which it does not accept in other protein, and this character is maintained over the seed. the concentration by these bile acids or its salt -- the anaclitic activation reaction of enzyme, and albumin concentration -- [the enzyme activity depressor effect over anaclitic LDL cholesterol and VLDL cholesterol] Since an opposite similar action is shown, this phenomenon has suggested existence of the interaction part which both commit competitively in a reaction with enzyme, LDL cholesterol, and VLDL cholesterol.
in this invention -- an enzyme reaction -- HDL cholesterol -- the HDL cholesterol concentration from immediately after the reaction start which passes in a plane 1 and whose rate of reaction is the largest since it goes on specifically -- anaclitic reaction variation is obtained. Therefore, having the outstanding character which is not seen is understood by the reaction characteristic [in / in this invention / a conventional method given / said / in a gazette] at this point. Moreover, by making one more or more sorts of auxiliary blocker live together suitably, for example, an usable pH condition can be made to be able to expand to the weak alkali side, or the amount of use albumin can be decreased. When shifting, it was clear from conditions suitable with a natural thing for singularity to also decrease, and for the reaction of LDL cholesterol and VLDL cholesterol to be overdue subsequently to HDL cholesterol, and to begin advance. Since Measurement Division by a number second bit is desired less than 1 minute in the simple test by the dry chemistry especially asked about the quick nature of measurement, the difference of demonstrate [by 1 liquid specification / simultaneously with a reaction start / singularity] in sufficient principle to accept an essential difference between this invention and a conventional method is clear.
. Hereafter, although a work example explains this invention concretely, these do not limit the range of this invention. Especially in the following work examples, unless it refused, cholesterol esterase of pancreas origin was used as cholesterol esterase.
Work example 1: Fractionation of the lipid fraction by an ultracentrifugal method Fractionation of the lipid fraction by an ultracentrifugal method was performed according to Akio Kudo's (arteriosclerosis, 6, 39, 1978) etc. procedure. 16mg of EDTA sodium salt, 4g of sucrose, 3.2g of potassium bromide, and 0.8g of sodium chloride were added to 16ml of pooled serum, and, specifically, it dissolved in it. Apart from this, three kinds of specific gravity liquid was created. That is, the specific gravity liquid of specific gravity 1.21 dissolved 20g of sucrose, 15g of potassium bromide, and 5g of sodium chloride in 100ml of purified water, and was prepared. It mixed with the specific gravity liquid of specific gravity 1.063, and it prepared said 30ml of specific gravity liquid and 70ml of purified water of specific gravity 1.21. Moreover, the specific gravity liquid of specific gravity 1.006 dissolved in 97.5ml of purified water, and prepared 2.5g of sucrose.
1.9ml of the above-mentioned processing serum was put into the centrifugation vessel of 10ml capacity, 0.8ml of specific gravity liquid of specific gravity 1.21 was calmly stratified with the syringe in this upper layer, and centrifugality of the centrifugation vessel was carried out at 50000rpm at 10 degrees C for 20 hours. After the end of centrifugal processing, although all the with a specific gravity of 1.21 or less lipid fractions gathered for the top layer part, 1.6ml of specific gravity liquid of specific gravity 1.063 and 2ml of specific gravity liquid of specific gravity 1.006 were further stratified on this top layer part.

Centrifugality of this centrifugation vessel was further carried out at 50000rpm for 4 hours, and each lipid fractions were collected.
After each fraction carried out overnight dialysis (under refrigeration), cold storage of it was carried out to the physiological salt solution.
Work example 2: The activation reaction by cholic acid sodium, and depressor effect by albumin 1 mM-ESPT, 0.5mM-4-AP, a 5microg [/ml] peroxidase, 1u/ml cholesterol oxidase, And 1.0ml of 40 mM-Bis-Tris buffer solution (pH 6.2) (buffer solution X is called hereafter) containing 0.25u/ml cholesterol esterase was warmed for 5 minutes at 37 degrees C. LDL fraction 10microl prepared in the work example 1 was added to this buffer solution X, and the variation per hour of coloring was recorded on it on the wavelength of 546nm. A result is shown in drawing 1 (the curve a of drawing 1). Most change of absorbance was not observed.
next, instead of [of said buffer solution X] -- said buffer solution X -- further -- same measurement was carried out using the buffer solution (buffer solution Y is called hereafter) containing 0.75mM cholic acid sodium. The curve b of drawing 1 shows a result. It turned out that change of absorbance is observed and the reaction of enzyme and cholesterol in an LDL fraction is activated with cholic acid sodium.
Then, same measurement was carried out instead of said buffer solution X using the buffer solution (buffer solution Z is called hereafter) which contains cow albumin in said buffer solution Y 0.8 more%. The curve c of drawing 1 shows a result. There is almost no change of absorbance and it turned out that the reaction of enzyme and cholesterol in an LDL fraction is inhibited by addition of albumin.
Work example 3: Reaction variation per hour Buffer solution Z1.0ml was warmed for 5 minutes at 37 degrees C. HDL fraction 20microl prepared in the work example 1, LDL fraction 10microl, VLDL fraction 10microl, or 10micro of normal pooled-serum l was added to this buffer solution Z as a sample, respectively, and absorbance change on the wavelength of 546nm was recorded on it.
A result is shown in drawing 2 . In drawing 2 , "HDL" shows the result at the time of using an HDL fraction as a sample. In a result when "LDL" uses an LDL fraction as a sample, a result when "VLDL" uses a VLDL fraction as a sample, and "serum" show the result at the time of using the normal pooled serum as a sample, respectively. Compared with the HDL fraction, the reaction prevention over cholesterol of an LDL fraction and a VLDL fraction was clear.
Work example 4: Preparation of reaction singularity (1) enzyme-reaction liquid with the serum lipid fraction by a reaction mixture object chromatography method 0.75ml of 40 mM-Bis-Tris buffer solution containing 1.3 mM-ESPT (pH 6.2) 10micro of pooled-serum l was added as a sample for (calling it A solution hereafter), and it warmed for 5 minutes at 37 degrees C. In this solution, 2mM-4-AP, a 20microg [/ml] peroxidase, 1.6% cow albumin, 3mM cholic acid sodium, 4u/ml cholesterol oxidase, and 0.25ml (B solution is called hereafter) of 40 mM-Bis-Tris buffer solution (pH 6.2) containing 1u/ml cholesterol esterase were added. By adding 70micro of 1M tris hydroxy methane solution l, and terminating an enzyme reaction, after warming for 10 minutes after warming for 5 minutes after adding B solution at 37 degrees C The reaction mixed-solution M1 (namely, thing warmed for 5 minutes after adding B solution) and the reaction mixed-solution M2 (namely, thing warmed for 10 minutes after adding B solution) were obtained. Apart from this, since 70micro of 1M tris hydroxymethyl aminomethane solution l is previously added to 0.25ml of B solution and the reaction stop was carried out as control before an enzyme reaction, what added 0.75ml of A solution (reaction mixed-solution Mc) was prepared. The reaction mixture object chromatography shown below analyzed these reaction mixed-

solutions. Said 100micro of reaction mixed-solutions 1 were used for each analysis.
(2) Analysis by reaction mixture object chromatography According to the procedure of W.Marz and others (Clin.Chem., 39/11, 2276-2281, 1993), the action of the lipid fraction in serum before and behind an enzyme reaction was investigated. Namely, the 0.1M-phosphate buffer solution which contains 0.2 M-NaCl using the column for gel filtration (Superose6 column: Pharmacia) (pH 7.4)
The sample was separated by the 0.3ml flow rate for /as an eluate. Next, after making the separated lipid fraction join the enzyme reagent for total measurement cholesterol (product made from YATORON) which is flowing by the flow rate for 70microl./and warming it for 5 minutes in a 37-degree C incubator, absorbance change was recorded on the wavelength of 580nm. It is the chromatogram [curve (b) of the reaction mixed-solution M2 to drawing 4 about the chromatogram [curve (c)] of the reaction mixed-solution Mc which is control before the chromatogram [curve (a)] of the reaction mixed-solution M1, and an enzyme reaction at drawing 3 .
]
And the chromatogram [curve (c)] of the reaction mixed-solution Mc which is control before an enzyme reaction is shown. In addition, the sample pooled serum is changed to purified water, and the chromatogram [curve (d)] of the example of a reagent blank test which operated like the reaction mixed-solution M1, and was prepared is collectively shown in drawing 4 .
What diluted with said eluate beforehand each lipid fraction prepared in the work example 1 100 times was poured in as a sample, and the elution position of each fraction determined it from the result. Each lipid fraction was eluted from VLDL of the amount of polymers according to the size as a lipid complex, and the peak appeared in order of LDL and HDL continuously.
In this way, in order that the acquired peak may show the total cholesterol contained in each lipid fraction, change of each accepted peak shows the singularity and reacting weight to each lipid fraction directly as a result of the enzyme reaction in a specimen. As shown in drawing 3 , the enzyme reaction for 5 minutes proved that the peak of VLDL and LDL also begins reduction further at the reaction for 10 minutes to only the peak of HDL having disappeared, as shown in drawing 4 . Therefore, in this invention, after HDL cholesterol reacted specifically, it was clear that LDL cholesterol and VLDL cholesterol begin a reaction.
Work example 5: Measurement of a real specimen 40 mM-Bis-Tris buffer solution (pH 6.2) [= reagent 1] 225microl which contains 1.3 mM-ESPT as a reagent constituent of this invention, 2mM-4-AP, a 20microg [/ml] peroxidase, 1.6% cow albumin, 3mM cholic acid sodium, 4u/ml cholesterol oxidase, And the reagent constituent which takes the composition of a 2 reagent system with 40 mM-Bis-Tris buffer solution (pH 6.2) 75microl [= reagent 2] containing 1u/ml cholesterol esterase was used, and it measured on 3micro of sample 1 conditions which use using the autoanalyzer. In 5 minutes, the reaction time with a reagent 1 also carried out reaction time with the continuing reagent 2 for 5 minutes, and measured on the dominant wavelength of 546nm, and the subwavelength of 700nm. Ten serum specimens carried out, compared and contrasted measurement by a sedimentation method (product made from the first chemistry) beforehand. Moreover, as a standard substance in both measuring methods, the standard serum for lipid measurement (welfare and product made from an iatrotechnique promotion meeting) was used.
The correlation of both measuring methods was shown in drawing 5 . this invention procedure showed the sedimentation method regularly used from the former, and good correlation, and it was checked that

measurement of the HDL cholesterol by this invention procedure is exact.
Work example 6: Relative reactivity of auxiliary blocker Each lipid fraction (every 10microl) which carried out fractionation to 0.75ml of 40 mM-Bis-Tris buffer solution (pH 7.0) containing 1 mM-ESPT in the work example 1 was added, respectively, and it warmed for 5 minutes at 37 degrees C. The 0.2% solution of each auxiliary blocker shown in 2mM-4-AP and the following table 1 at this, a 20microg [/ ml] peroxidase, 0.
0.25ml of 40 mM-Bis-Tris buffer solution (pH 7.0) which contains cow albumin, 1.7u/ml cholesterol esterase, and 4u/ml cholesterol oxidase 09% was added, and the absorbance in the wavelength of 546nm was measured.
Reactivity of n-OTG to each lipid fraction was made into 100%, and the relative reactivity of each compound was searched for. A result is shown in Table 1. In addition, the indicated value over VLDL and LDL is a reaction prevention rate. Under the conditions by this example, it turned out that other compounds have a prevention rate comparable as n-OTG to VLDL and LDL as shown in Table 1. On the other hand, since the reaction singularity over HDL was accepted, it was checked that it can be adapted in this invention procedure.

表1

補助ブロッカー	VLDL (%)	LDL (%)	HDL (%)
n－OTG	100	100	100
n－HTG	100	99	41
SM－1000	99	98	81
CHAPS	99	99	63
CHAPSO	99	94	83
n－ODG	100	100	41
n－HDG	100	99	42
MEGA－8	100	99	23
MEGA－9	100	100	32
MEGA－10	100	99	23

Work example 7: In the case of a reaction with an albumin addition effect (1) LDL fraction Lipid fraction LDL10microl prepared in the work example 1 as a sample was added to 0.75ml of 40 mM-BES buffer solution (pH 7.0) containing 1.3 mM-ESPT, and it warmed for 5 minutes at 37 degrees C. To this, 3mM cholic acid sodium, 2mM-4-AP, 0.3% albumin, A 20microg [/ml] peroxidase, 0.2%n-OTG, 1.7u/ ml cholesterol esterase, and 0.25ml of 40 mM-BES buffer solution (pH 7.0) containing 4u/ml cholesterol oxidase were added, and absorbance change on the wavelength of 546nm was recorded. Moreover, as a

comparative experiment, except not adding albumin, the same operation as the above was carried out and the absorbance (wavelength of 546nm) change was also recorded. Those results are shown in drawing 6 . In drawing 6 , "addition" shows the case where albumin is added and "additive-free" shows the case where albumin is not added. It was checked that the reaction of enzyme and cholesterol of an LDL fraction is efficiently checked by addition of albumin.

(2) In the case of a reaction with serum 0.08% cow albumin, 0.05%n-OTG, 1 mM-ESPT, 0.5mM-4-AP, a 5microg [/ml] peroxidase, 0.75mM cholic acid sodium, 0.13u/ml cholesterol esterase, and 40 mM-Bis-Tris buffer solution containing 1u/ml cholesterol oxidase (pH 6.8)

1.0ml was warmed for 5 minutes at 37 degrees C. Beforehand, about three serum (Serum A - serum C) which measured the total cholesterol value (the product made from YATORON, enzymatic process), and the HDL cholesterol value (the product made from the first chemistry, sedimentation method), 10microl was added to this, respectively and absorbance change on the wavelength of 546nm was recorded on it. Moreover, it recorded instead of said buffer solution similarly about the same serum using the solution except cow albumin from said buffer solution. Moreover, serum was replaced with purified water and the example of a reagent blank test was also recorded.

A result is shown in drawing 7 . In Serum A, the 200 mg/dl and 66 mg/dl; serum B was [198 mg/dl and 93mg/dl;, and Serum C of the total cholesterol value and HDL cholesterol value of serum which were measured beforehand] 109 mg/dl and 38 mg/dl, respectively. In drawing 7 , curvilinear (1) - (3) is an albumin additive-free case, and curvilinear (4) - (7) is the case of albumin addition. A curve (1) and (5) are the results of Serum A, and a curve (2) and (4) are the results of Serum B. Moreover, a curve (3) and (6) are the results of Serum C. A curve (7) is an example of a reagent blank test. It was clear that absorbance change is shown on the conditions which added albumin according to an HDL cholesterol value from a reaction start.

Work example 8: Reaction singularity with the serum lipid fraction by the reaction mixture object chromatography method in the conditions which use auxiliary blocker 40 mM-Bis-Tris buffer solution containing 1.3 mM-ESPT (pH 6.8) (D solution is called hereafter) 10micro of pooled-serum 1 was added to 0.75ml as a sample, and it warmed for 5 minutes at 37 degrees C. To this, 2mM-4-AP, a 20microg [/ml] peroxidase, 0.2%n-OTG, It added and 0.36% cow albumin, 3mM cholic acid sodium, 4u/ml cholesterol oxidase, and 0.25ml of 40 mM-Bis-Tris buffer solution (pH 6.8) (E solution is called hereafter) containing 0.5u/ml cholesterol esterase were warmed for 5 minutes. 70micro of 1M tris hydroxymethyl aminomethane solution 1 was added to this, the reaction was stopped, and the reaction mixed-solution M3 was obtained. Moreover, same operation was carried out from E solution using the solution except cow albumin instead of said E solution, and the reaction mixed-solution M4 was obtained to it. Furthermore, since 70micro of 1M tris hydroxymethyl aminomethane solution 1 is previously added to 0.25ml of E solution and a reaction stop is carried out as control before an enzyme reaction, it is D solution 0.

What added 75ml (reaction mixed-solution Mo) was prepared.

Each reaction mixed-solution was analyzed with the reaction mixture object chromatography method shown in the work example 4. [drawing 8 / the chromatogram [curve (c)] of the reaction mixed-solution Mo which is control before the chromatogram [curve (a)] of the reaction mixed-solution M3, and an enzyme reaction] The chromatogram [curve (c)] of the reaction mixed-solution Mo which is control before the chromatogram [curve (b)] of the reaction mixed-solution M4 and an enzyme reaction is shown in drawing 9 . In addition, as a sample, the pooled serum is changed to purified water and the

chromatogram [curve (d)] of the example of a reagent blank test which operated like the reaction mixed-solution M3, and was prepared is collectively shown in drawing 8 .

The reaction when adding albumin proved that the peak of VLDL and LDL also begins reduction further at the albumin additive-free reaction to only the peak of HDL having disappeared. Therefore, it was clear under auxiliary blocker existence addition's of albumin to inhibit the reaction of LDL cholesterol, VLDL cholesterol, and enzyme, and to make it react specifically only with HDL cholesterol.

Work example 9: Measurement of the real specimen at the time of using auxiliary blocker as a reagent constituent of this invention 40 mM-Bis-Tris buffer solution (pH 7.0) [= reagent 1] 225microl containing 1.3 mM-ESPT, 2mM-4-AP, a 20microg [/ml] peroxidase, 0.2%n-OTG, 0.09% cow albumin, 3mM cholic acid sodium, 4u/ml cholesterol oxidase, And it measured on 3micro of sample l conditions which use using automatic part chip box equipment using the reagent constituent which takes the composition of a 2 reagent system with 40 mM-Bis-Tris buffer solution (pH7.O) [= reagent 2] 75microl containing 1u/ml cholesterol esterase. In 5 minutes, the reaction time with a reagent 1 also carried out reaction time with the continuing reagent 2 for 5 minutes, and measured on the dominant wavelength of 546nm, and the subwavelength of 700nm. 76 serum specimens carried out, compared and contrasted measurement by a sedimentation method (product made from the first chemistry) beforehand. Moreover, as a standard substance in both measuring methods, the standard serum for lipid measurement (welfare and product made from an iatrotechnique promotion meeting) was used. The correlation of both measuring methods is shown in drawing 10 . this invention procedure showed the sedimentation method regularly used from the former, and good correlation, and it was checked that measurement of the HDL cholesterol by this invention procedure is exact.

Work example 10: Measurement of the real specimen in a dry type system of measurement as a reagent constituent of this invention 5mM ESPT, 2mM 4-AP, a 1mg [/ml] peroxidase, 4% cow albumin, 3mM cholic acid sodium, 1.4u/ml cholesterol oxidase, And 40mM containing 0.76u/ml cholesterol esterase Bis-Tris buffer solution (pH 6.2) is used, on the transparent base material (product made from polystyrene), the spot of said 20micro of reagent constituents l was carried out, and they were dried. 5micro of samples l were carried on the dryness reagent constituent, and it is on an aluminum block for 8 minutes, and warmed at 37 degrees C. The coloring thing was measured by reflective type densitometer analysis.

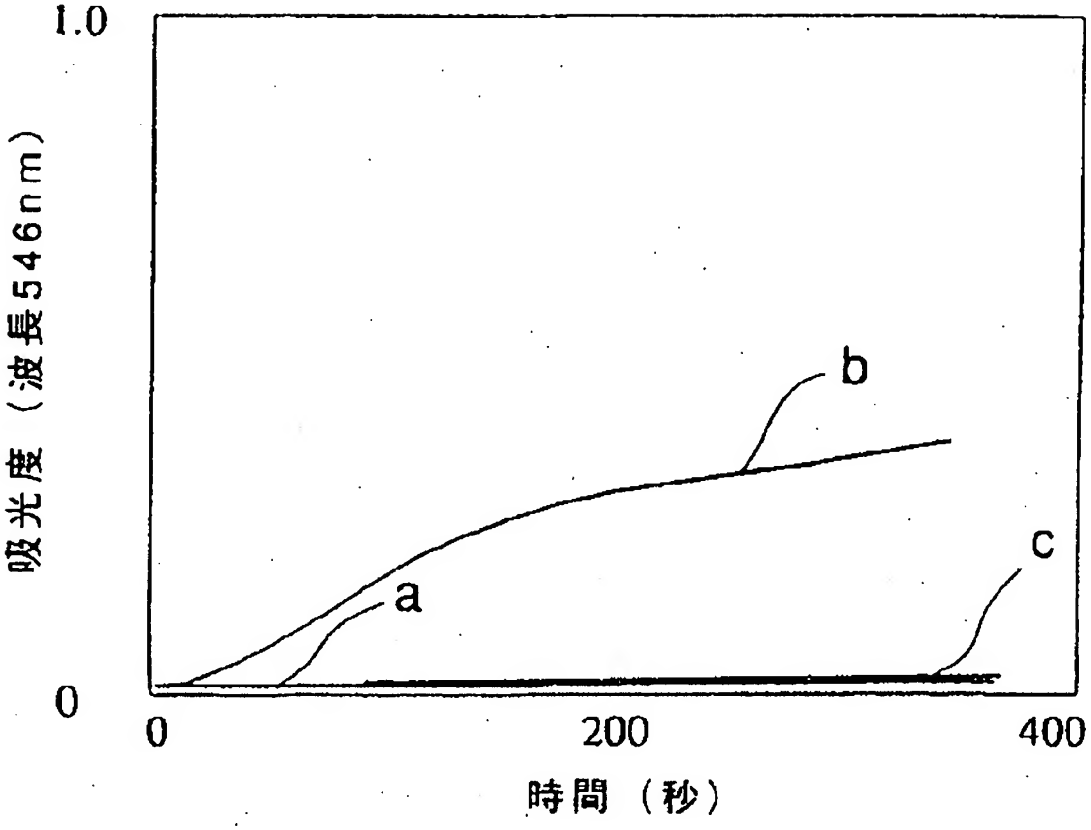
19 serum specimens compared and contrasted beforehand by performing measurement by a sedimentation method (product made from the first chemistry). Moreover, as a standard substance in both measuring methods, the standard serum for lipid measurement (welfare and product made from an iatrotechnique promotion meeting) was used. The correlation of both measuring methods was shown in drawing 11 . this invention method inclined with the sedimentation method regularly used from the former, and showed =0.97 and section =-6.1 mg/dl, coefficient-of-correlation =0.902, and good correlation, and it was checked that measurement of the HDL cholesterol by this invention procedure is exact.

Industrial availability Highly precise measurement of HDL cholesterol can be performed by simple operation, without performing centrifugal operation of a sample (serum or plasma).

As mentioned above, although this invention was explained about the specific mode, modification obvious to a person skilled in the art is included in this invention.

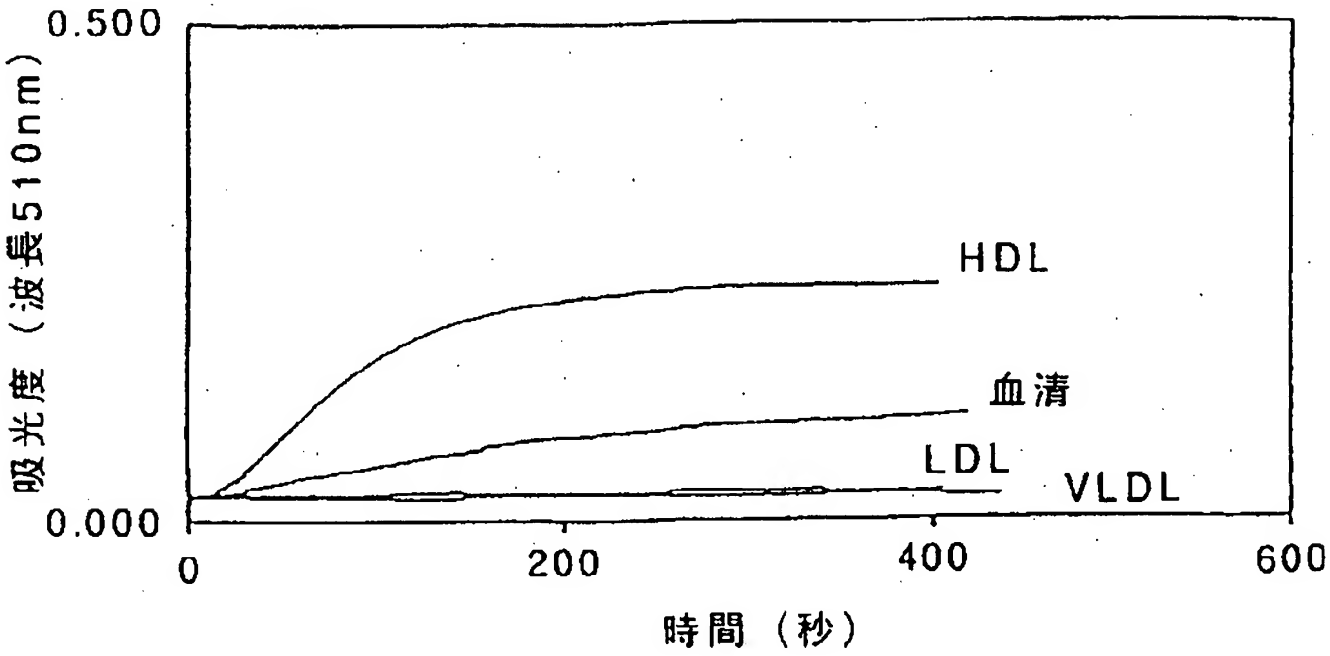
[Drawing 1]

FIG. 1



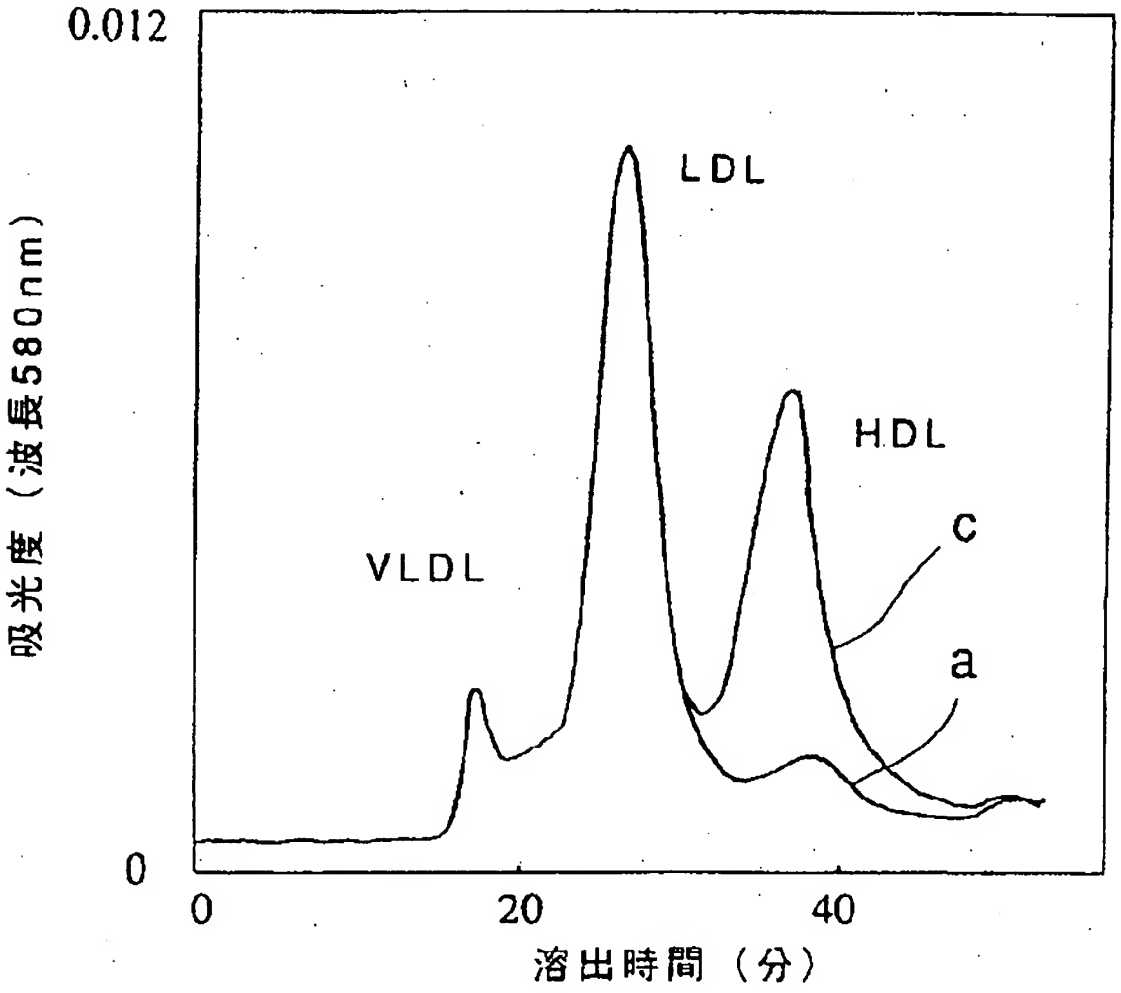
[Drawing 2]

FIG. 2



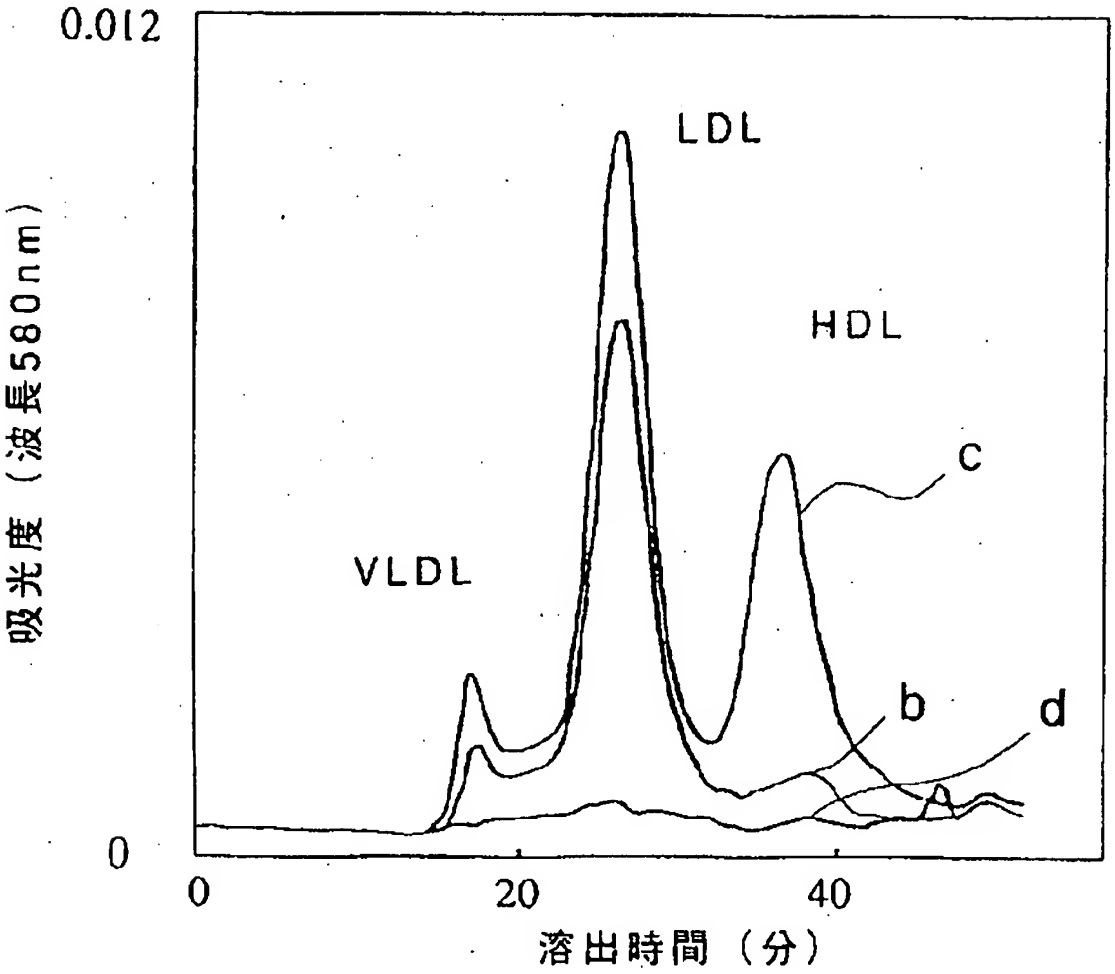
[Drawing 3]

FIG. 3



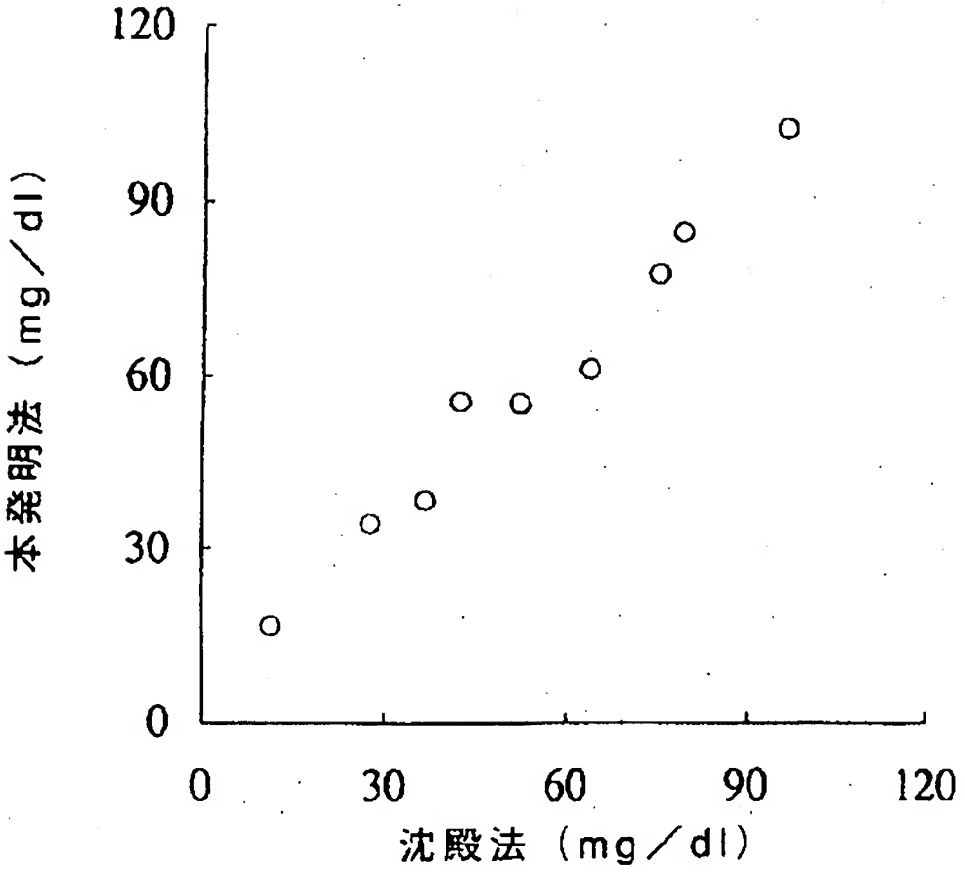
[Drawing 4]

FIG. 4



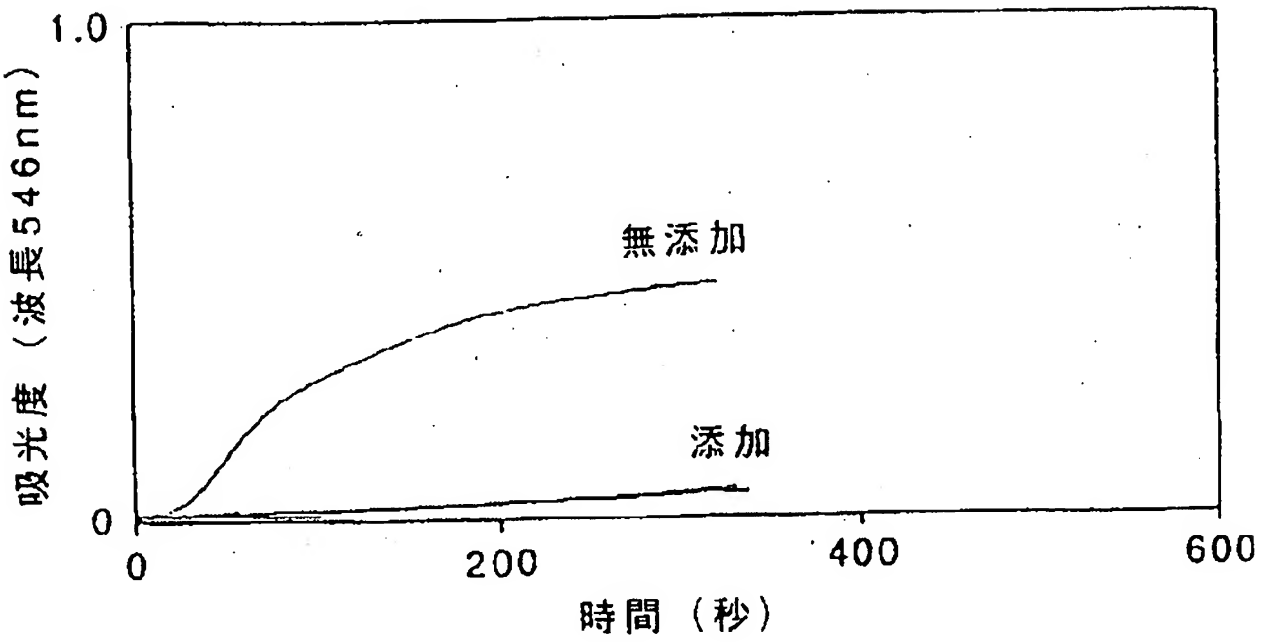
[Drawing 5]

FIG. 5



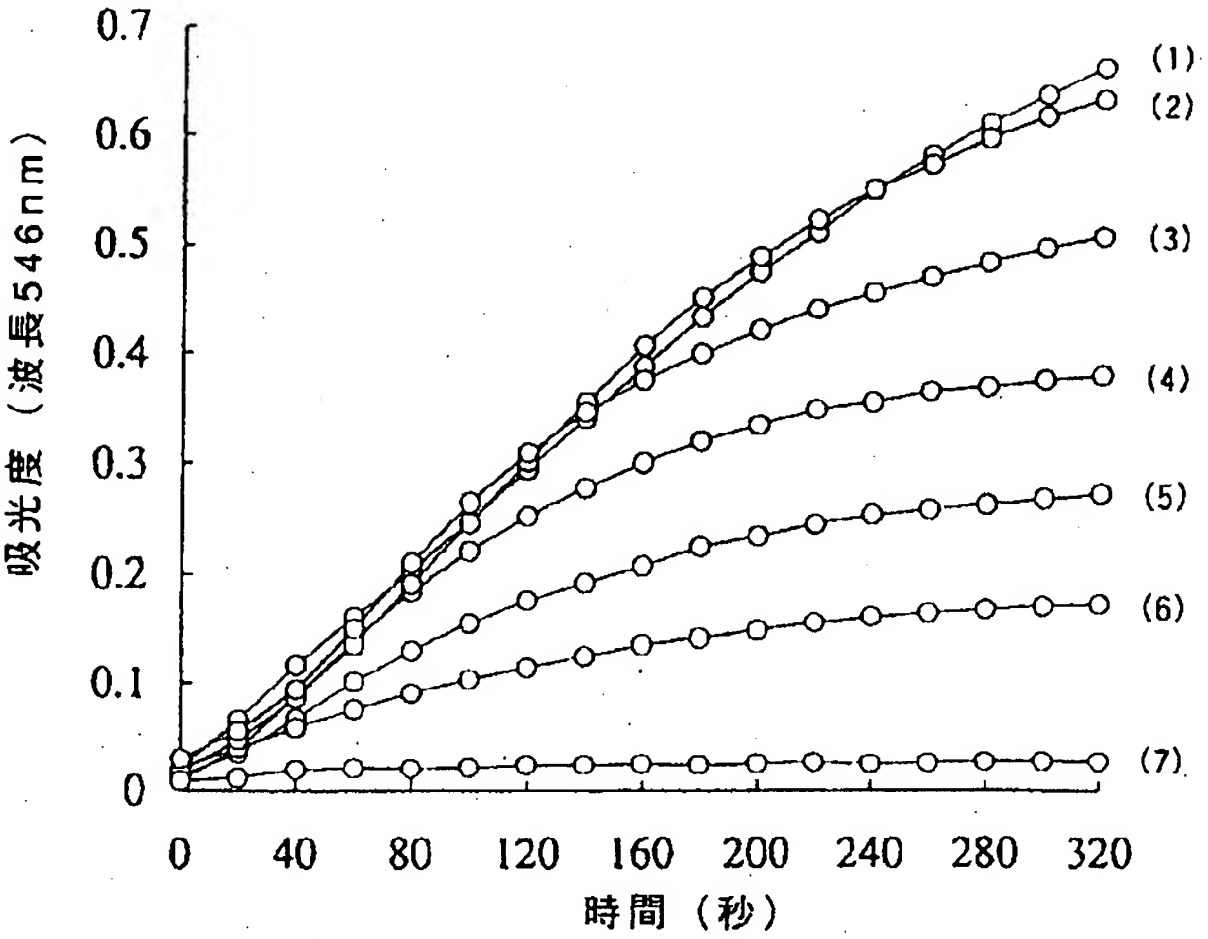
[Drawing 6]

FIG. 6



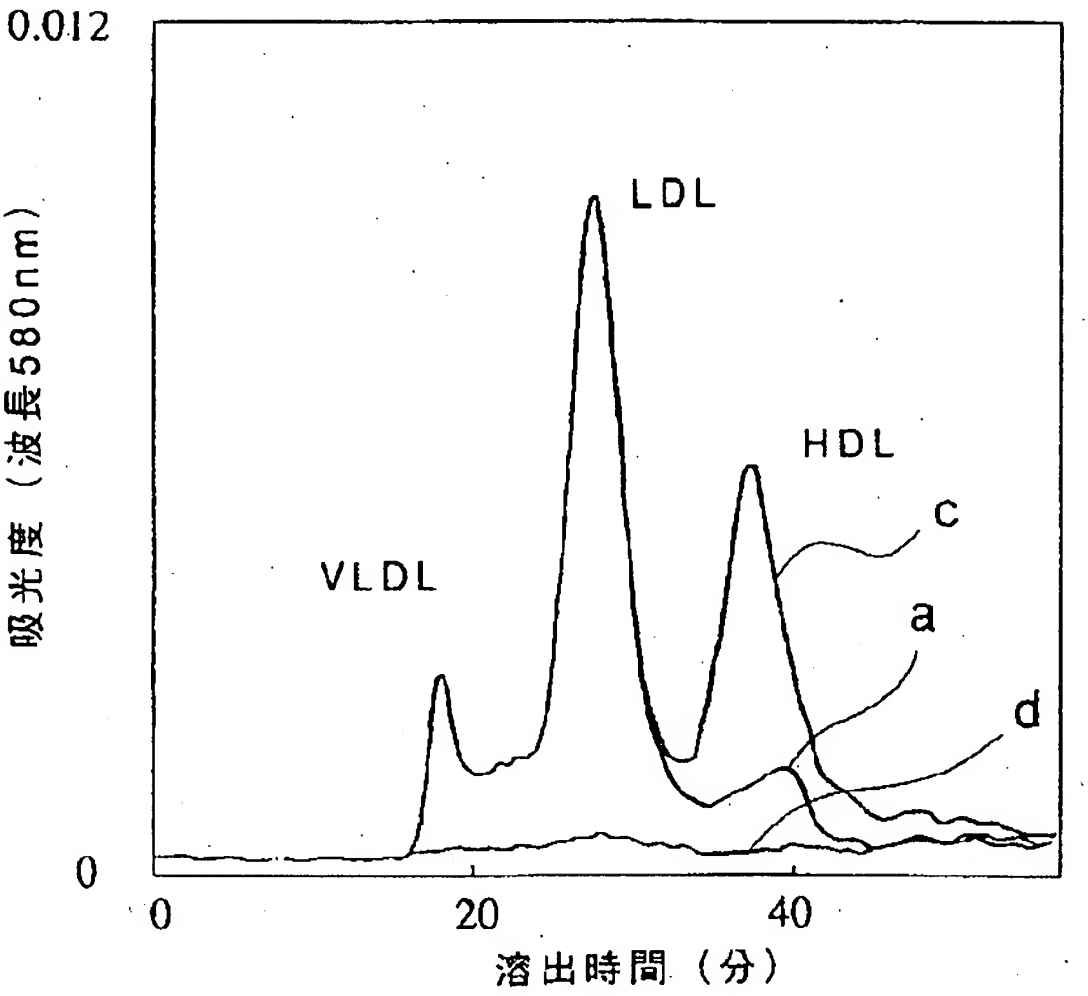
[Drawing 7]

FIG. 7



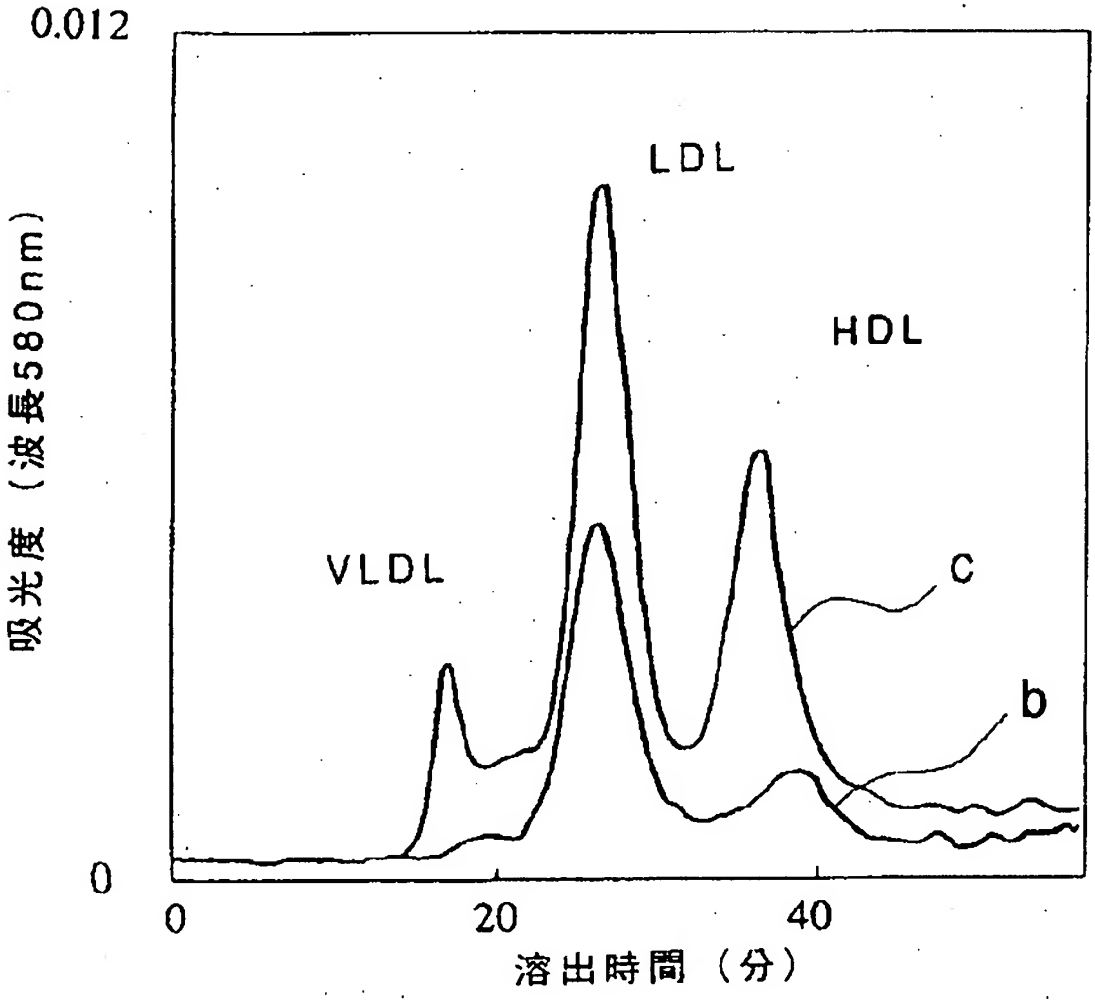
[Drawing 8]

FIG. 8



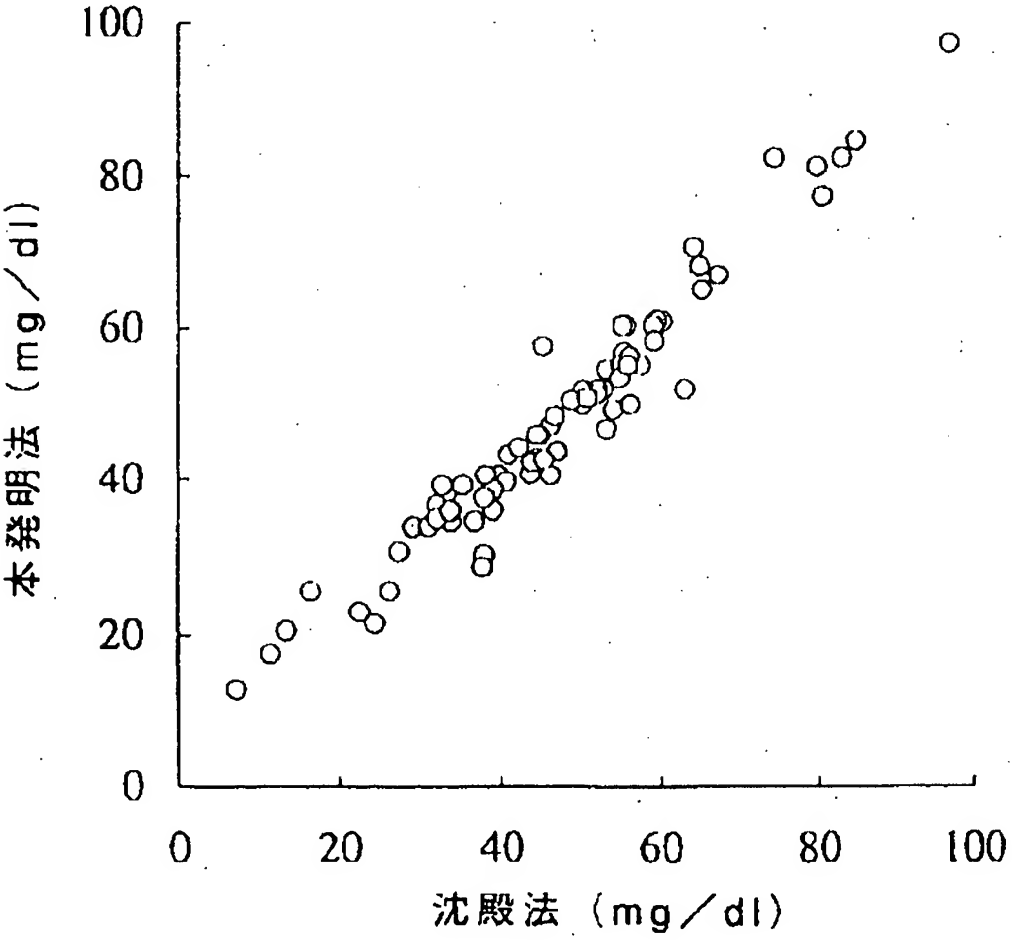
[Drawing 9]

FIG. 9



[Drawing 10]

FIG. 10



[Drawing 11]

FIG. 11